Cancer testis antigens and immunosurveillance in human cutaneous squamous cell and basal cell carcinomas

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Abstract: PURPOSE: Non melanoma skin cancer (NMSC) is the most common cancer and comprises basal cell (BCC) and squamous cell carcinoma (SCC). The incidence of SCC increases drastically in immunosuppressed individuals, suggesting a critical role of the immune system in controlling SCC. To find an explanation for the selective immunosurveillance of SCC, we investigated the expression of cancer-testis antigens (CTA), MHC class I and the infiltration by immune cells in BCC and SCC. Experimental design: We determined the expression of 23 different CTA in 63 BCC and 40 SCC biopsies of immunocompetent and in 20 biopsies of immunosuppressed SCC patients by RT-PCR and immunohistochemistry. IgG responses to 36 tumor antigens were measured by Western Blotting and ELISA. MHC-I expression and CD8+ T cell infiltration were analyzed by immunohistochemistry in BCC and SCC of immunocompetent and immunosuppressed patients and in imiquimod-treated BCC patients.RESULTS: We found expression of at least one CTA in 81% of BCC and in 40% of SCC. We did not detect CTA-specific serum IgG. Most SCC, but not BCC, expressed MHC-I and were infiltrated with CD8+ cells. Imiquimod-treated BCC expressed MHC-I and were infiltrated by CD8+ T cells.CONCLUSIONS: We propose that immunosurveillance controls SCC, but not BCC, as the latter lacks MHC-I. This fits with the increased incidence of SCC in immunosuppressed individuals and may explain the relatively low prevalence of CT-antigen expression in SCC as a result of CD8+ T cell driven immunoediting.

DOI: https://doi.org/10.1158/1078-0432.CCR-09-3136

Clinical Cancer Research

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Clin Cancer Res Published OnlineFirst June 2, 2010.

Published OnlineFirst on June 2, 2010 as 10.1158/1078-0432.CCR-09-3136

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Running Title

Immunosurveillance and CT-antigen expression in NMSC

Key Words: SCC, BCC, CT-antigens, immunosurveillance

Statement of translational relevance

Non Melanoma Skin Cancer (NMSC) is the most common malignancy worldwide. Although surgery is a successful standard of care, it often brings disfiguration and pain to the patients. To investigate alternative treatment options like immunotherapy, we have shown for the first time that CT-antigens are expressed in over 50% of NMSC patients. These tumor antigens are absent from healthy tissues except testis and placenta and are currently under investigation as cancer vaccine targets in numerous clinical trials.

Also, we present the novel finding that imiquimod upregulates MHC class I expression in BCC, which is accompanied by infiltrating $CD8^+$ cells.

Taken together these data can lead to new immunotherapy approaches of especially unresectable NMSC and open new application ranges for the immune response modifier imiquimod, which can possibly be translated to other malignancies.

Abstract

Purpose: Non melanoma skin cancer (NMSC) is the most common cancer and comprises basal cell (BCC) and squamous cell carcinoma (SCC). The incidence of SCC increases drastically in immunosuppressed individuals, suggesting a critical role of the immune system in controlling SCC. To find an explanation for the selective immunosurveillance of SCC, we investigated the expression of cancer-testis antigens (CTA), MHC class I and the infiltration by immune cells in BCC and SCC.
Experimental design: We determined the expression of 23 different CTA in 63 BCC and 40 SCC biopsies of immunocompetent and in 20 biopsies of immunosuppressed SCC patients by RT-PCR and immunohistochemistry. IgG responses to 36 tumor antigens were measured by Western Blotting and ELISA. MHC-I expression and $CD8^+$ T cell infiltration were analyzed by immunohistochemistry in BCC and SCC of immunocompetent and immunosuppressed patients and in imiquimod-treated BCC patients.
Results: We found expression of at least one CTA in 81% of BCC and in 40% of SCC. We did not detect CTA-specific serum IgG. Most SCC, but not BCC, expressed MHC-I and were infiltrated with $CD8^+$ cells. Imiquimod-treated BCC expressed MHC-I and were infiltrated by $CD8^+$ T cells.
Conclusions: We propose that immunosurveillance controls SCC, but not BCC, as the latter lacks MHC-I. This fits with the increased incidence of SCC in immunosuppressed individuals and may explain the relatively low prevalence of CT-antigen expression in SCC as a result of $CD8^+$ T cell driven immunoediting.

Introduction

Non melanoma skin cancers (NMSC) are the most common cancers in the Caucasian population and include squamous (SCC) and basal cell carcinoma (BCC). According to the WHO, 2-3 million new cases of NMSC occur globally each year[1]. NMSC presents a low metastatic potential, however, the tumor can cause substantial local damage if not treated early. The standard surgical care of SCC and BCC often causes severe pain and disfiguration (recently reviewed in (1)).

The risk to develop NMSC significantly increases up to 250 fold in immunosuppressed patients, such as organ transplant recipients (OTR). In addition, aggressive and metastatic variants of SCC develop in OTR, which represent a critical health burden (2, 3). The ratio BCC:SCC is 4:1 in immunocompetent patients, but 1:10 or higher in OTR, suggesting a critical role for immunosurveillance in SCC but not in BCC. However, studies explicitly addressing this issue are lacking.

Although NMSCs are frequently infiltrated by immune cells (for review see (4)), the immune system often seems incapable of eradicating the tumor. Downregulation of E-Selectin and recruitment of regulatory T cells (T reg) (5) as well as malfunctioning intratumoral myeloid dendritic cells (6) have been proposed as mechanisms that compromise local tumor-specific immunity in SCC. In the case of BCC, MHC-I absence or down-regulation (7, 8) and the presence of T regs (9) was observed.

Immunotherapy is an alternative treatment approach, especially for cancers that can't be surgically removed due to their site or to multiple metastases. Usually, patients are immunized with tumor-associated antigens in order to induce or boost tumor-specific immunity and this approach has shown objective clinical responses in some patients. Many of those vaccines contain tumor-specific differentiation antigens

[1] http://www.who.int/uv/faq/skincancer/en/index1.html

(Melan-A/MART-1, tyrosinase, gp100 in the case of melanoma), overexpressed antigens (e.g. p53, survivin) or cancer-testis (CT) antigens (e.g. NY-ESO-1, members of the MAGE family) (10, 11). CT-antigens belong to an extended family of antigens that are expressed by a large variety of malignancies and that are absent from healthy tissue except for testis and placenta. In addition, cancer patients often develop spontaneous immune responses towards CT-antigens, which illustrates their immunogenicity (for review see (12)). In contrast to other malignancies including melanoma, tumor-specific immune responses and tumor-associated antigens that may be the target of such responses are scarcely investigated in NMSC and as a consequence, it is unknown whether immunotherapy may be a suitable therapeutic modality for these cutaneous malignancies. In a recent study, the expression of the CT-antigen MAGE-A4 was identified in cutaneous SCC on an immunohistochemical level (13), but no other data regarding the expression and immunogenicity of CT-antigens in BCC and SCC are available.

In this paper we present the first wide screening for CT-antigen expression in SCC and BCC in immunocompetent and –suppressed (OTR) patients. Furthermore, when comparing BCC and SCC with respect to MHC class I expression and infiltration by $CD8^+$ T cells, we found that both immunologically relevant parameters were significantly lower in BCC. This may offer an explanation for the selective increase of SCC-incidence in OTR.

Topical application of imiquimod, a Toll-like receptor (TLR) 7 agonist, results in total regression of the lesion in a large proportion of NMSC patients. Interestingly, we found a significant upregulation of MHC class I expression in BCC upon imiquimod treatment, which adds another possible mode of action to the presumed ones, including stimulation of innate immunity and the induction of apoptosis in tumor cells (14, 15).

Materials and Methods

Patient samples

All patients enrolled in the study were treated at the Dermatology Department of the University Hospital of Zurich. The study was approved by the cantonal ethics committee (EK No. 1017). All patients signed informed consent. Specimens of patients receiving imiquimod treatment had been published previously (15, 16). 8 patients were analyzed, which applied 5% imiquimod cream (Aldara, 3M Pharmaceuticals, Saint Paul, MN) once daily 5 times per week for a maximum of 6 weeks. Patients were evaluated weekly until the tumor began to show signs of erosion. At this point, the tumor was surgically excised and stored in formalin.

For PCR extraction, primary cutaneous SCC (including the non-invasive forms actinic keratosis and morbus bowen to represent the whole facet of the disease), BCC and healthy skin samples were obtained during MOHs micrographic surgery and frozen in liquid nitrogen. We obtained 113 biopsies from immunocompetent patients (Suppl. table 1) and 20 SCC biopsies from organ transplant recipients receiving immunosuppressive treatment (Suppl. table 2), histological classification was done according to (17).

From 23 immunocompetent BCC and 2 OTR BCC and 8 immunocompetent SCC and 1 OTR SCC patients, serum was collected.

Cell culture

BCC biopsies were minced into pieces ≤ 2mm^2 and digested in Collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 1-2hrs at 37°C. Fragments were digested with 0.25% Trypsin (Invitrogen, Carlsbad, CA) for 15-30 min. Debris was filtered through 100μm and 40 μm nylon filters (BD Biosciences, San Jose, CA) and single cells were cultured in Keratinocyte SFM Medium (Invitrogen, Carlsbad, CA) with 0.5U/ml Penicillin (Invitrogen, Carlsbad, CA) and 0.5μg/ml Streptomycin (Invitrogen, Carlsbad, CA). Upon reaching confluency, cells were trypsinized and cultured as described above.

***In vitro* imiquimod and IFN-γ treatment**

BCC short-term cultures (passage 1) were incubated in Keratinocyte SFM Medium (Invitrogen, Carlsbad, CA) containing either 100U/ml IFN-γ (Pepro Tech Inc., Rocky Hill, NJ) or 30μM imiquimod (Aldara Crème 5%, MEDA Pharma, Wangen-Brütisellen, Switzerland) for 36 hours at 37°C.

Flow cytometry

Samples were stained with FITC-anti-β2-microglobulin, APC-anti-HLA-A,B,C (BD biosciences, San Jose, CA) and LIVE/DEAD violet fixable dead cell stain kit (Invitrogen, Carlsbad, CA). Samples were measured on a Cyan ADP (Beckman Coulter, Fullerton, CA) and analyzed with Flow Jo Software (Tree Star, Ashland, OR).

RNA isolation and reverse transcription PCR (RT-PCR)

Author Manuscript Published OnlineFirst on June 2, 2010; DOI: 10.1158/1078-0432.CCR-09-3136
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Total RNA/DNA extraction from frozen samples was performed using the AllPrep DNA/RNA Mini Kit (Quiagen, Valencia, CA) and RNA was subjected to DNAse I digestion (Invitrogen, Carlsbad, CA). Total RNA concentration and purity was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). 150 ng of RNA were transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Foster City, CA).

RT-PCR was carried out using Taq DNA Polymerase (NEB, Ipswich, MA) with primers specific for the individual CT-antigens or for β-actin as internal control (Supplementary table 3). The cDNA samples were amplified using an Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) using the following conditions: An initial denaturation step for 10 min at 95°C, 35 amplification cycles (denaturation for 1 min at 95°C, annealing for 1 min at variable temperatures (Supplementary table 3), elongation for 1 min at 72°C) and a final elongation step for 10 min at 72°C. All primers were tested on at least five independent control healthy skin samples. All kits were used according to manufacturers' recommendations.

Immunohistochemistry

Paraffin embedded tissue sections were stained with mouse-anti human monoclonal antibodies against CD8 (1:100; DAKO A/S, Glostrup, Denmark), CD56 (1:50; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), CT-45/KiA10 (1:50, generous gift from H. J. Heidebrecht, University of Kiel, Germany), MAGE-A1 (1:200, generous gift from the Ludwig Institute for Cancer Research, New York, NY), MHC-I (1:1000, RDI Research Diagnostics, Inc., Concord, MA), NY-ESO-1 (1:50, ZYMED Laboratories Inc., South San Francisco, CA), rabbit-anti-human polyclonal antibody against PRAME (5μg/ml, Abcam limited, Cambridge, MA) and the mouse hybridoma

supernatant 57B (13) that predominantly recognizes MAGE-A4 (undiluted, generous gift by Prof. Giulio C. Spagnoli, Department of Surgery, University Hospital Basel, Switzerland). Primary antibodies were detected using the ultraVIEW DAB detection kit (Ventana Medical Systems, Tucson, AZ). Sections were counterstained with hematoxylin, dehydrated and mounted. All sections were stained with the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) using Ventana reagents for the entire procedure.

Image analysis and quantification

Images of the stained paraffin sections were acquired on a Zeiss Axiophot HAL100 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with the KYF70 digital camera (JVC, Yokohama, Japan) and the software analySIS^D (Olympus, Tokyo, Japan). Adobe Photoshop Version 10.0 (Adobe systems incorporated, San Jose, CA) was used for image analysis.

Intensity of immunoreactivity was scored in arbitrary units as negative (0), weakly (1), moderately (2) or strongly (3) positive as illustrated in Figure S1. This panel of figures was compiled before the systematic evaluation of the tumor cohort was commenced and served as reference. Quantification of $CD8^+$ cells present in intra- and peritumoral regions as well as invasive front were acquired counting cells in high power fields (HPF) of 40x magnification. In case of a heterogeneous histology, up to 3 HPF were counted and the average is displayed in the figures. A pathologist advised on and supervised the evaluation of immunohistochemistry.

Western blot analysis

We tested sera from 34 patients for the presence of CT-antigen specific IgG by Western Blotting as described (18), using the recombinant proteins CT-45, NY-ESO-1 and PRAME. All proteins were overexpressed in *E. coli*, NY-ESO-1 was produced as previously described (18), CT-45 was a generous gift from H. J. Heidebrecht, University of Kiel, Germany and PRAME was a generous gift from C. Melief, University of Leiden Medical Center, The Netherlands.

ELISA

ELISA screening was carried out as recently described (Gnjatic *et al.*, 2009). 34 NMSC sera were screened for the presence of IgG specific responses for 36 different tumor antigens (for detailed list of antigens see Fig. S2).

Statistical analysis

Statistical analysis was performed with SPSS, version 17.0 (SPSS Inc, Chicago, IL, USA). P values < 0.05 were considered significant. The t-Test, Wilcoxon Test, Fisher´s Exact Test and Pearsons Correlations were used for analysis, depending on the data set.

Results and Discussion

CT-antigens are broadly expressed in non-melanoma skin cancer (NMSC)

We investigated expression of 23 CT-antigens in biopsies from 113 immunocompetent NMSC patients and 13 corresponding healthy tissues with reverse transcription PCR (RT-PCR) and immunohistochemistry.

Overall, CT-antigens were broadly expressed in NMSC on the mRNA level, PRAME was the most frequently expressed antigen with 55% overall expression in NMSC, followed by MAGE-A4 (25.6%), MAGE-A9 (23.9%) and NY-ESO-1 (14.2%). We found no expression of non-X chromosome-linked CT-antigens like Boris or BRDT (Figure 1) and no expression of any CT-antigen in the healthy skin samples.

We did not observe any difference in CT-antigen expression between non-invasive and invasive SCC, hence the various histological types are presented together as one group (SCC) in all the figures.

Analysis of CT-antigen expression by immunohistochemistry on paraffin sections confirmed our RT-PCR results in all cases investigated (Figure 1C). Most CT-antigens were homogeneously expressed within the entire tumor, however, the expression of MAGE-A4 and PRAME varied in expression intensity between patients and often a stronger staining was observed in cells detaching from the tumor structure, so-called acantholytic cells (Figure 1C).

CT-antigens are frequently co-expressed in various malignancies (19-21). Our results in non-melanoma skin cancer confirmed these observations, as over 40% of NMSC biopsies co-expressed ≥2 antigens and we observed examples of co-expression of as much as 11 antigens (Figure 2C).

All CT-antigens were more frequently expressed in BCC than in SCC, except for MAGE-A3 (Fig. 1B). In addition, BCC expressed more antigens per biopsy on average than SCC (Figure 2). The ratio of SCC to BCC in patients is usually 1:4, which means that BCC represents 80% of non-melanoma skin cancer and SCC represents 20%. However, in patients under immunosuppressive treatment (OTR), the ratio of SCC to BCC is reversed to be 10:1, meaning that BCC represents only approximately 10% of non-melanoma skin cancer in this population of patients, whereas over 90% are SCC. This makes SCC the most common cutaneous malignancy in OTR (3, 22), suggesting a role for adaptive immunity in the control of SCC. Immunological pressure in SCC may explain why the percentage of CT-antigen$^+$ biopsies as well as the number of CT-antigens per biopsy is lower in SCC compared to BCC (23). However, we did not observe a significant difference in CT-antigen mRNA expression in SCC from immunocompetent patients compared to SCC from OTR, with respect to the number of CT-antigens per biopsy or to the percentage of biopsies expressing 1 or more CT-antigens (Figure 3). This observation had previously been made for MAGE-A4 (13). This finding may be unexpected at first glance, but may be explained as follows: The majority of OTR develop SCC within the first 5 years after start of immunosuppressive treatment (3), which argues against *de novo* formation of the disease and in favor of uncontrolled outgrowth of subclinically preexisting malignancies. Before immunosuppressive treatment removed immunological control and allowed pathogenic outgrowth of the malignancy, subclinically existing SCC were presumably subjected to immunological pressure, resulting in selective outgrowth of tumors that express fewer CT-antigens.

No spontaneous humoral immune responses to CT antigens in NMSC patients

CT-antigens often cause spontaneous humoral responses in patients with cutaneous malignancies like melanoma (18, 24) and cutaneous T-cell lymphoma (25). We used western blotting and ELISA to investigate whether also NMSC patients display humoral immune responses to tumor antigens.

We screened 34 sera from NMSC patients (9 SCC, 25 BCC) for antibodies against an array of CT-antigens by western blot (data not shown) and ELISA (Figure S2). The absence of tumor antigen-specific IgG in sera from patients with NMSC was in contrast with data from most other malignancies and was therefore unexpected. We explain the virtual absence of tumor-specific humoral immune responses by the relatively small tumor load of NMSC in comparison to other tumor entities. It has been well documented, that titers of tumor-specific IgG increase with progressive disease (26, 27) *i.e.* with increasing tumor load and/or the occurrence of metastases, both of which are rare in NMSC.

Low expression of MHC class I and paucity of infiltrating $CD8^+$ cells in BCC suggests limited immunosurveillance

The selective increase of the SCC risk in immunosuppressed individuals suggests a more pronounced role of immunosurveillance in SCC than in BCC. One possible explanation may be the relative absence of suitable tumor-associated antigens in BCC, however, our results show the opposite (Fig 1-2). A relative absence of MHC class I molecules on BCC, which precludes recognition by CT-antigen-specific $CD8^+$ effector T cells, may be another possibility. We thus compared the expression of MHC class I in BCC and SCC and correlated the amount of infiltrating $CD8^+$ cells to

MHC-I expression by immunohistochemistry. We found that both the expression of MHC class I and the infiltration of the invasive front, peri- and intratumoral regions by $CD8^+$ cells were drastically reduced in most BCC biopsies when compared to SCC biopsies (Fig. 4 and 5), which confirms previous observations (7, 8). Both the expression of MHC class I as well as the number of infiltrating $CD8^+$ cells was most prominent in the invasive front of SCC biopsies and statistical analysis revealed a significant correlation between these two parameters (Fig. 5C). Thus, we propose that the relative absence of MHC class I molecules from tumor cells and the ensuing absence of infiltrating $CD8^+$ cells in BCC makes this malignancy comparably resistant to the adaptive immune response. Natural killer cells were virtually absent from both BCC and SCC (assessed by CD56 staining, data not shown).

Nevertheless, although MHC-I staining does not differ between SCC OTR and SCC patients, the number of $CD8^+$ cells in the intra-, peri- and invasive regions of the tumor are decreased in SCC OTR compared to immunocompetent patients (Fig. 5B). This coincides with a recent study showing an overall decrease of cytotoxic T cells in OTR (28) and further outlines the role of $CD8^+$-mediated immune control of SCC and probably not of BCC. These results fit the fact that the activated sonic hedgehog pathway presumably is the driving force in BCC formation (for review see (29)) and that immunological tumor defence therefore has a minor impact on BCC formation.

Local treatment with imiquimod results in upregulation of MHC class I and increased peritumoral $CD8^+$ T cell infiltration in BCC

Repeated topical application of imiquimod, a TLR7-agonist, results in regression of superficial skin cancers, presumably through the induction of innate immunity and

apoptosis of tumor cells (14, 15). To investigate the possibility that imiquimod treatment impacts on the adaptive tumor-specific immune response, we compared the expression of MHC class I molecules and the presence of $CD8^+$ T cells in BCC biopsies before and after imiquimod treatment.

The expression of MHC class I by tumor cells was significantly stronger in biopsies after imiquimod treatment, which was accompanied by an increase of peritumoral $CD8^+$ T cells (Fig. 6). As BCCs do not express TLR7 (30, 31), the observed upregulation of MHC class I molecules on tumor cells presumably is an indirect effect of imiquimod, which may be mediated through type I and type II interferons produced by infiltrating plasmacytoid dendritic cells (pDCs) (16) or by tumor stroma, or through stimulation of tumoricidal activity of inflammatory myeloid DCs and pDCs (32). Our assumption that up-regulation of MHC class I by imiquimod does not result from a direct interaction with BCC is supported by our observation that *in vitro* treatment of BCC cultures with 30μM imiquimod does not upregulate surface expression of HLA class I molecules. In contrast, treatment with IFN-γ (100U/ml) induced increased surface expression of β2-microglobulin as well as HLA-A,B,C molecules at least by two-fold (assessed by measuring mean fluorescence intensity by flow cytometry, data not shown). Alternatively, treatment with imiquimod may induce changes in the microenvironment other than upregulation of MHC class I molecules that support local tumor-specific $CD8^+$ T cells, as has been reported for SCC (5, 33). As the $CD8^+$ T cells of imiquimod-treated BCC patients remain in the peritumoral region and do not seem to penetrate the BCC nests (Fig. 6), their tumor-antigen specificity and anti-tumor effector functions remain to be confirmed for BCC.

Taken together, we found that BCC expresses no or low levels of MHC class I molecules, and that – probably as a result thereof – significantly lower numbers of

$CD8^+$ immune effector cells infiltrate the tumor. We thus propose that BCC is less subjected to immunological pressure than SCC, and our finding that BCC express more immunogenic CT antigens as well as the fact that the incidence of SCC but not of BCC increases in individuals under immunosuppressive treatment support this hypothesis. Furthermore, we propose that the therapeutic effect of local imiquimod treatment may involve - besides stimulation of the innate defense and induction of tumor cell apoptosis - the upregulation of MHC class I on tumor cells and the concomitant influx of $CD8^+$ cells.

Acknowledgements

We thank Claudia Matter, Alexandro Landshammer and Christa Dudli-Furrer for excellent technical assistance, Günther Hofbauer for discussion, help with the manuscript and providing material and Mirjana Maiwald-Urosevic for samples of imiquimod-treated patients.

This work was supported by a grant by the Ludwig Institute for Cancer Research/Cancer Research Institute (Cancer Antigen Discovery Collaborative), Atlantic Philanthropies, the Cancer Research Institute, the Hanne Liebermann Foundation, the Hartman Müller Foundation, the Terry Fox Foundation and the Zürcher Krebsliga. Marjam J. Barysch and Reinhard Dummer were supported by funds obtained from the Swiss National Foundation (grant no. 310040-103671) and the Gottfried and Julia Bangerter Rhyner Stiftung.

Figure Legends

Fig. 1. CT-antigen expression in biopsies from immunocompetent NMSC patients as determined by RT-PCR. 1A. Expression of investigated CT-antigens in total number of biopsies (n=113). 1B. Differential expression of antigens in BCC (n=73) and SCC (n=40).1C. CT-antigen expression of selected SCC and BCC samples as determined by immunohistochemistry.

Fig. 2. CT-antigen expression in biopsies from immunocompetent NMSC patients as determined by RT-PCR. 2A. Average of CT-antigens expressed per biopsy. 2B. Percentage of patients expressing at least one CT-antigen. 2C. Co-expression of CT-antigens in biopsies from immunocompetent NMSC patients (n=113).

Fig. 3. CT-antigen expression in biopsies from immunocompetent (SCC) vs. immunosuppressed (SCC OTR) SCC patients as determined by RT-PCR. 3A. Percentage of patients expressing at least one CT-antigen. 3B. Average of CT-antigens expressed per biopsy.

Fig. 4. Immunohistochemical staining of MHC-I (A and B) and CD8 (C and D) on consecutive sections of BCC Z-T-468 (B and D) and SCC Z-T-509 (A and C).

Fig. 5. Quantification of MHC class I expression and infiltration of $CD8^+$ T cells in biopsies from NMSC patients by immunohistochemistry. 5A. Histology (H)-score of MHC-I in SCC, BCC, SCC of immunosuppressed organ transplant recipients (OTR) and BCC of OTR. 5B. Quantification of $CD8^+$ counts per 40x high power field (HPF) in intra- and peritumoral regions and invasive front of SCC, BCC, SCC OTR and BCC

OTR. 5C. Correlation between H-score for MHC-I expression with CD8+ T cell counts per HPF in sections from immunocompetent SCC patients. 5D. Correlation between H-score for MHC-I expression with $CD8^+$ T cell counts per HPF in sections from immunocompetent BCC patients.

Fig. 6. Quantification of MHC class I expression and infiltration of $CD8^+$ T cells in imiquimod-treated and non-treated BCC by immunohistochemistry. 6A. H-score of MHC-I staining in biopsies from BCC patients taken before (pre) and after (post) imiquimod treatment (n=7). 6B. Quantification of $CD8^+$ counts per 40x high power field (HPF) in intra- and peritumoral regions of biopsies from BCC patients before (pre) and after (post) imiquimod treatment (n=8). 6C. MHC-I expression and correlating $CD8^+$ infiltration in sections from one representative BCC patient before (pre) and after (post) imiquimod treatment.

Fig. S1. IHC scoring sheet for MHC-I expression as used as a reference for expression analysis. Numbers 0-3 indicate intensity of staining as illustrated in the pictures.

Fig. S2. Summary of antibody titers obtained in ELISA with 36 antigens. Titers depicted in bold: significant reaction (>100). Titers depicted in italics: sera tested only in screening phase. ¹ Sera considered „sticky“ and therefore non-specific. ² Sera from OTR.

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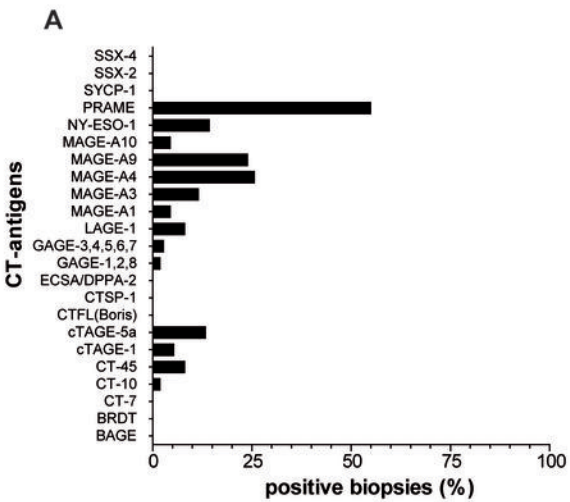
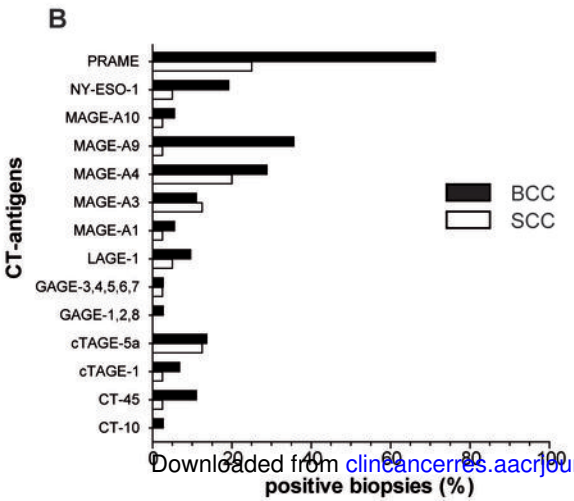
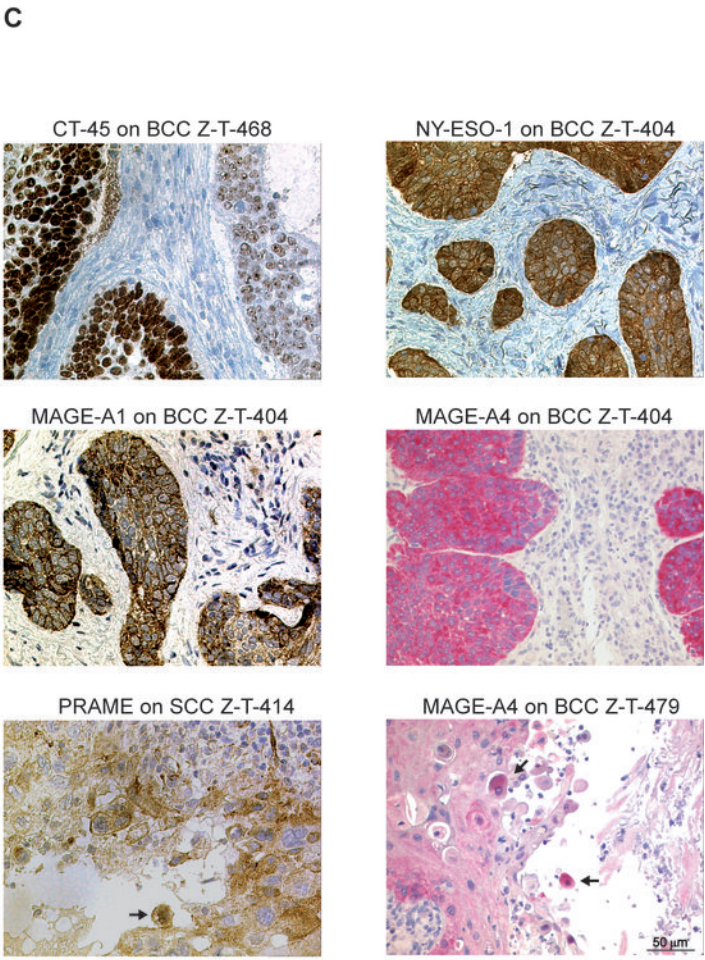
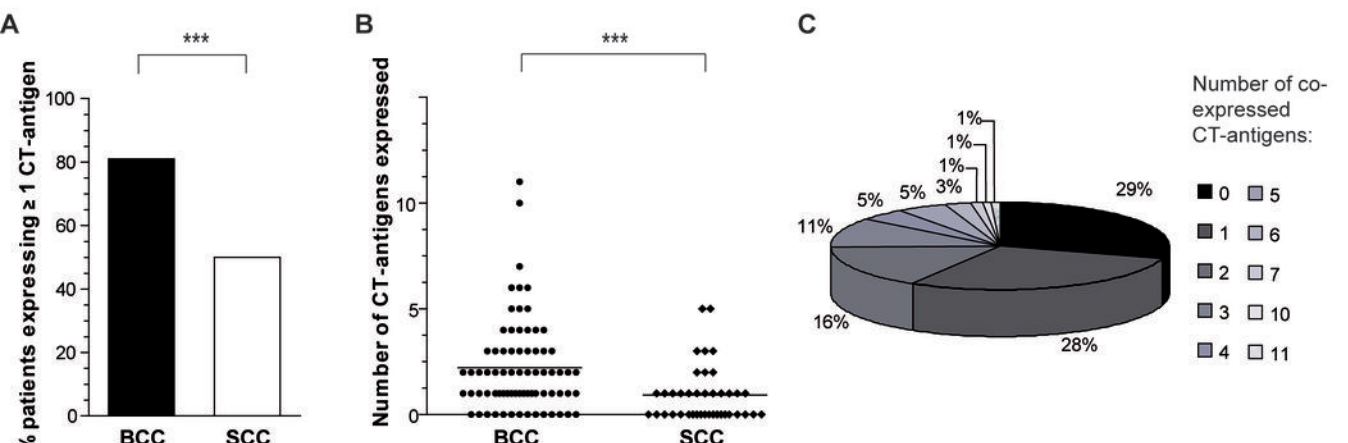


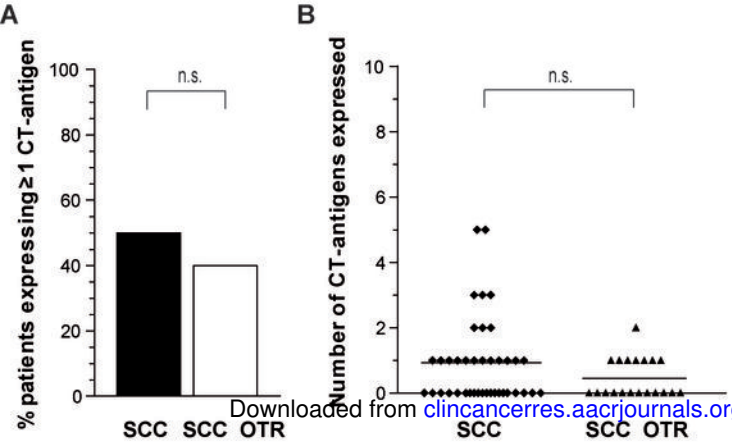




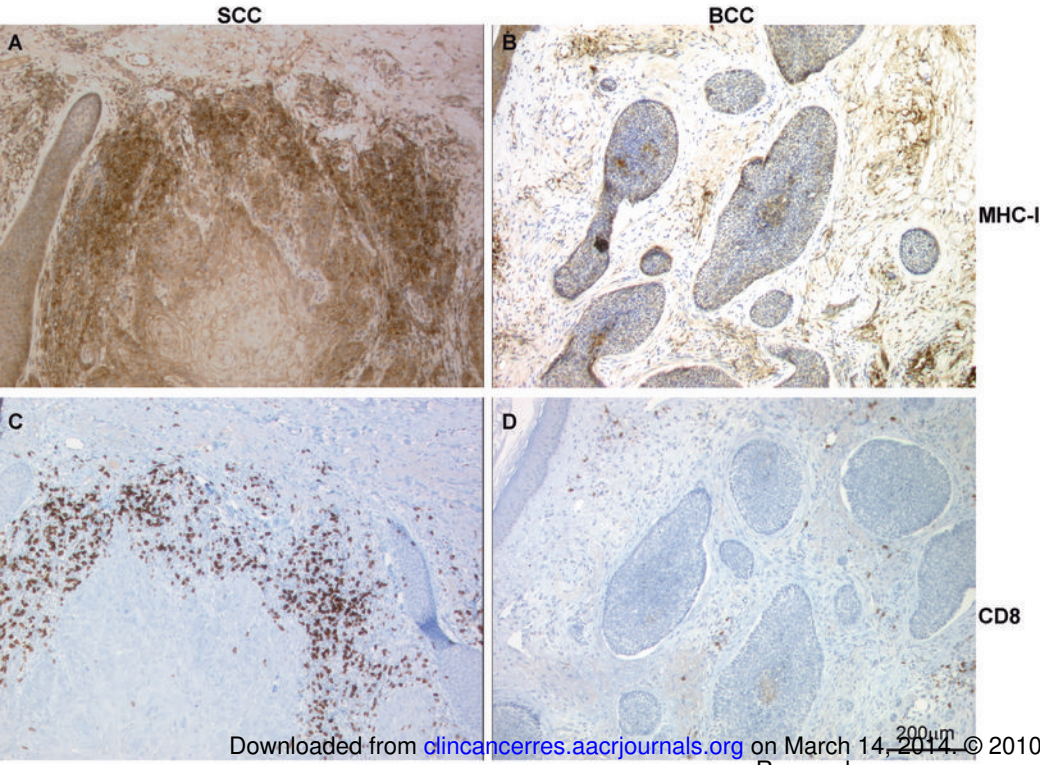
Walter *et al.*, Fig.2



Walter *et al.*, Fig.3



Walter *et al.*, Fig. 4



Walter *et al*, Fig. 5